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SUMMARY

Viruses play important roles in population dynamics and as drivers of evolution in single-celled marine phytoplankton. Viral infection of *Ostreococcus tauri* often causes cell lysis, but two spontaneously arising resistance mechanisms occur: resistant cells that cannot become infected and resistant producer cells that are infected but not lysed, and which may slowly release viruses. As of yet, little is known about how consistent the effects of viruses on their hosts are across different environments. To measure the effect of host resistance on host growth, and to determine whether this effect is environmentally dependent, we compared the growth and survival of susceptible, resistant and resistant producer *O. tauri* cells under five environmental conditions with and without exposure to *O. tauri* virus. While the effects of exposure to virus on growth rates did not show a consistent pattern in populations of resistant cells, there were several cases where exposure to virus affected growth in resistant hosts, sometimes positively. In the absence of virus, there was no detectable cost of resistance in any environment, as measured by growth rate. In fact, the opposite was the case, with populations of resistant producer cells having the highest growth rates across four of the five environments.

INTRODUCTION

Marine viruses play a large role in nutrient and energy cycling in the oceans. Viral lysis of single celled organisms releases large quantities of organic matter into the environment, making nutrients available for use by bacteria and algae. This process has been termed the viral shunt (Wilhelm and Suttle, 1999). Studies on marine viruses typically focus on the importance of viruses in nutrient cycling and the release of organic matter through cell lysis. Despite the important role of marine viruses in ecosystem function across many environments, from nutrient rich coastal waters to more oligotrophic regions of the open ocean (Bruussard, 2004), host-virus interactions are typically studied in single environments. Here, we use the *Ostreococcus tauri*/*Ostreococcus tauri* virus model system to investigate variation in host-virus interactions across environments to understand (1) whether susceptibility/resistance to viruses changes with environmental change, and (2) whether the growth effect of host resistance depends on environmental context or resistance type.

We explore the relationship between host responses to environmental change and the resistance strategies of those hosts using the marine picoeukaryote *Ostreococcus tauri* (order Mamiellales). *O. tauri* is commonly isolated from Mediterranean lagoons that are connected to the open ocean via narrow channels (Clerissi *et al.*, 2014). These channels limit the exchange of seawater between the lagoon and ocean, making variations in the environmental salinity, pH, temperature and nutrients more extreme than in the open ocean (Bellec *et al.*, 2010; Clerissi *et al.*, 2014). *Ostreococcus tauri* viruses (OtVs) have been sampled frequently in water samples collected from lagoon and coastal waters where *O. tauri* is found. OtVs have strict host specificity (Clerissi

62 *et al.*, 2012), and the three OtVs sequenced to date have all been described as lytic
63 viruses (Derelle *et al.*, 2008; Weynberg *et al.*, 2009, 2011). Virus infection of *O. tauri*
64 usually causes cell lysis in susceptible (S) cells, though two mechanisms of resistance
65 have been observed (Thomas *et al.*, 2011). In the first case, viruses are unable to
66 infect the host, and these cells are referred to here as resistant (R). In the second case,
67 hosts are tolerant to viral infection and are able to slowly release them without
68 damage to the host cell. These cells are termed resistant producers (RP). In this paper,
69 we refer to the three cell types as resistance types.
70
71 Resistance type could have consequences for growth and other cell properties, such as
72 size and chlorophyll content. For example, a trade-off of acquiring resistance to viral
73 lysis may come as a fitness cost. This often occurs as reduced competitive ability
74 (Lenski, 1988; Bohannan *et al.*, 2002) and sometimes reduced growth rate (Lennon *et*
75 *al.*, 2007; Frickel *et al.*, 2016). A modification in cell surface receptors to limit virus
76 attachment could also result in a loss of the original function of the protein, such as
77 metabolism or being able to target the host immune system. In several bacteria
78 species, loss of a bacteriophage receptor results in lower virulence of the bacteria in
79 its host, thereby lowering the fitness of resistant compared to non-resistant strains
80 (Seed *et al.*, 2012; León and Bastías, 2015). Lastly, strong resistance to one specific
81 virus strain may lead to increased susceptibility to lysis by other strains, as has been
82 observed in *O. tauri* (Clerissi *et al.*, 2012) and cyanobacteria (Marston *et al.*, 2012;
83 Avrani and Lindell, 2015).
84
85 The group of viruses that infects phytoplankton is the Phycodnaviruses. These viruses
86 have been studied under environmental conditions that differ from a benign control

environment in a single driver, such as increases in temperature (Nagasaki and Yamaguchi, 1998; Wells and Deming, 2006), nutrient (Bratbak *et al.*, 1993, 1998; Bellec *et al.*, 2010; Clerissi *et al.*, 2014), light (Bratbak *et al.*, 1998; Weinbauer, 2004), UV (Jacquet and Bratbak, 2003), CO_2 (Larsen *et al.*, 2007; Chen *et al.*, 2014; Maat *et al.*, 2014) and pH levels (Weinbauer, 2004). When environmental conditions are stressful, one consequence can be inactivation of the virus particle. This affects host-virus interactions by preventing infection through structural degradation, the inability of the virus to inject its genome into the host or the inability of the virus to replicate (Børsheim, 1993; Jacquet and Bratbak, 2003). Additionally, since viral replication and life cycle are often closely linked to host metabolism, environmental changes such as increased temperature or nutrients will often have an indirect effect on responses to viral attack (Weinbauer, 2004; Danovaro *et al.*, 2011). Understanding the role of viruses in marine communities requires investigating their activity across environments. Here, we focus on the environmental changes of increased temperature, decreased nutrients, decreased light and decreased salinity levels.

Previous studies of resistance in *O. tauri* found that when each resistance type was maintained separately there was no significant difference in growth rates, such that a cost of resistance was too low to be detected by differences in growth alone. However, when resistant types were competed against each other, a competitive hierarchy was observed in which S had the fastest growth rate, followed by R and then by RP (Thomas *et al.*, 2011). Since the three resistance types share the same starting genotype, it is possible to make direct comparisons between them. In this study, an experiment was performed in which three populations of each *O. tauri* resistance type (S, R and RP) derived from a common ancestor were grown for one

week in the following environments in both the absence and presence of OtV5: high temperature, low light, low phosphate, and low salt. These environments were selected to represent relatively small variations from the control environment in which the populations are normally maintained in the laboratory, so that the cells responded, but were still able to grow at a rate that was measureable. The average number of cell divisions per day over a single transfer cycle (7 days), cell size and cell chlorophyll content were measured in the novel environments in the absence of OtV5. Offspring production over a fixed period of time is a proxy for fitness in single celled organisms in batch culture experiments (Brennan and Collins, 2015). Cell size and chlorophyll content were measured as additional phenotypes, to examine effects on organismal function other than cell division rates, since only small differences in growth were detected previously (Thomas *et al*., 2011). After one week of growth in the novel environment, all populations were inoculated with OtV5 and cell densities were measured three days after inoculation to test for susceptibility to viral lysis.

RESULTS

The effect of viral exposure on cell division rates depends on resistance type

After one week of growth in a novel environment, all populations were inoculated with OtV5 and cell densities were measured three days later. Supporting Information Tables S1 and S2 provide all statistical outputs in this study. Susceptibility of *O. tauri* to OtV5 was driven by resistance type, as expected (ANOVA, resistance type × virus treatment, $F_{2,234} = 360.14$, $p < 0.0001$). After inoculation of *O. tauri* with OtV5, all R and RP cells remained resistant to lysis and S cells remained susceptible (Figure 1). Thus, OtV5 inoculation had a significant effect on cell density (ANOVA effect of virus treatment on growth, $F_{1,234} = 361.62$, $p < 0.0001$), since populations of S cells fell to almost zero (Figure 1). No difference was observed in resistance between R and RP populations (t=0.46, p=0.66).

Counter to our expectation, the effect of virus inoculation did not vary with environment (ANOVA, environment × virus treatment, $F_{4,234} = 0.89$, $p < 0.46$). However, environment alone had a significant effect on growth ($F_{4,234} = 26.01$, $p < 0.0001$), because of the S cell lysis in all environments. Additionally, an interaction was identified between resistance type and environment (ANOVA, environment × resistance type, $F_{8,234} = 6.09$, $p < 0.0001$). For both R and RP cells, there were cases where virus inoculation resulted in higher growth rates than the non-inoculated controls (Figure 1). Cell densities were repeatedly higher in one inoculated population (NG'13) than the control in the low salt environment in R cells and in the low light environment for one population (NG27) in RP cells. This indicates that cell growth

153 can increase in response to viruses in resistant populations. This occurs consistently in
154 all replicates of a given population when it happens, but does not occur in all
155 populations of a resistance type. We also see cases where lysis in some populations of
156 S cells is incomplete, notably in the low light (NG'2) and low salt (NG'3)
157 environments. Again, this does not occur in all populations, but it occurs reliably in
158 replicates of the same population. While these effects of environment on lysis are not
159 statistically significant because they do not occur over all populations within a
160 resistance type, it could have evolutionary and ecological effects on the occasions
161 when it does occur, which we discuss below.
162
163 *Growth rate varied across environments regardless of resistance type*
164
165 All populations were grown in a novel environment in the absence of OtV5 for one
166 week, over which growth rate was measured. The response of *O. tauri* growth to the
167 environment depended on resistance type (effect of environment × resistance type, $F_{8,}$
168 $_{114}$=4.45, p=0.0001). Additionally, regardless of resistance type, population growth
169 rates differed between environments (effect of environment on growth $F_{4,114}$ = 231.39,
170 P<0.0001) (Figure 2). Growth rates were higher in the control environment except for
171 a single RP population, NG'10, which divided rapidly in the low salt environment
172 (Figure 2). Populations grown in the low phosphate environment all had reduced
173 growth rates and showed less variation in growth than in all other environments.
174
175 *The effect of resistance type on growth depends on environment*
176

Resistance type alone did not significantly affect the growth rate of *O. tauri* ($F_{2,6}$=2.88, p=0.1328). This is because S and R cells had similar population growth rates in all environments (Figure 2). In contrast, some populations of RP had different growth rates than both R and S cells. There was variation in growth rates between replicate populations of RP cells, with some populations consistently showing elevated growth rates. Two out of the three RP populations (NG'10 (shown by circle in Fig 2) and NG'16 (shown by cross in Fig 2) had higher growth rates than S and R cells in four out of the five environments ($F_{3,5}$=17.19, p=0.046). The single exception was the low phosphate environment, where all resistance types had similar low growth rates. These data indicate that there is either no cost or an undetectable cost of resistance in terms of growth to either infection or lysis over a range of environments, and that there can be a growth benefit of being resistant to lysis in some environments, as evidenced by the rapid growth of some RP populations. The low or absent cost of resistance is consistent with previous studies in single environments, which have reported costs of resistance detectable in competitions, but too low to be detectable by comparing growth rates (Thomas *et al.*, 2011).

Populations resistant to lysis can have a growth advantage in some environments

In order to assess whether the S, R, and RP resistance types responded similarly to the different environments, environments were ranked from best to worst, based on population growth rates. All resistance types displayed highest growth rates in the control environment (See Supporting Information Table S3). R cells had the same rank order of environments as the S cells. Since the growth rates of the RP cells were highly variable relative to the other resistance types, containing two populations that

grew quickly, the RP populations were grouped into fast growing (NG'10 and NG'16) and normal growing (NG27). RP cells showed the same rank order of environments for both the fast and normal growing populations, except in low salt for the fast growing populations. This was due to one population (NG'10) displaying exceptionally high growth. Growth rate was the same in the low salt and low light environments for the normal growing RP population. Fast growing RP cells had higher cell growth in all environments except low phosphate.

To measure how sensitive growth rates were to environmental change, the slopes of the ranked environments were compared (Figure 3). The two fast growing RP populations had a higher intercept (ANOVA effect of rank on growth, $F_{1,125}$=1112.56, p <0.0001), demonstrating the increase in growth rate compared to the other populations. These data show that faster growing populations had a stronger preference for environments in which they can grow more quickly, however in the lowest ranking environment (which was low phosphate for all resistance types), these populations grew equally badly.

Size and chlorophyll content vary between cells with different resistance types in response to environment

After one week of growth in a novel environment without viruses, cell size and relative cell chlorophyll content were measured. Response of resistance type on cell size depended on environment (effect of environment × resistance type, $F_{8,114}$=5.48, p <0.0001). Regardless of resistance type, environment had a significant effect on cell size ($F_{4,114}$=77.93, p <0.0001). Cells were larger under low light (t=3.83, p=0.0002)

and low phosphate conditions (t=7.49, p<0.0001), compared to the control environment (Figure 4). No significant effect of resistance type was observed on cell size ($F_{2,6}$=0.01, p=0.9945). However, under low phosphate, there was a large variation in cell size between the fast and normal growing RP populations.

The two fast-growing RP populations had smaller cells than the normal growing RP population in the low phosphate environment. The RP population with normal growth had cells that were similar in size to the S populations (Figure 4). To examine whether fast growing RP populations had different cell sizes than did populations with normal growth rates, *post hoc* models were used to analyse the two fast growing populations separately. Overall, no significant effect of resistance type was observed on cell size when normal and fast growing RP populations were analysed separately (ANOVA $F_{2,6}$=0.22, p=0.8812). Additionally, a model examining growth rate as a fixed effect was also performed. This showed a significant effect of growth rate ($F_{1,99}$=54.23, p<0.0001) and an interaction between resistance type and growth rate ($F_{2,99}$=4.64, p=0.01), although no effect of resistance type alone was detected ($F_{2,6}$=0.001, p=0.99). However, the statistical power in this data set, which contained only one population of normal growing RP cells and two populations of fast growing RP cells, was low, such that the chances of detecting an effect of resistance type on cell size is unlikely here even if one exists (power=0.142).

The effect of resistance type on chlorophyll content per cell volume depended on environment (effect of environment × resistance type, $F_{8,114}$=10.68, p <0.0001). In addition, environment alone had a significant effect on relative chlorophyll per cell volume ($F_{4,114}$=120.45, p <0.0001), however resistance type alone did not ($F_{2,6}$=1.61,

252 p =0.2757). Under low light, chlorophyll varied little between the three resistance
253 types. In the other environments, S and R strategies usually displayed similar
254 chlorophyll content levels with RP displaying lower chlorophyll levels in all
255 environments except low phosphate.
256
257 By inspection, we see that the fast growing RP populations have less chlorophyll per
258 cell volume than the normal growing RP population in all environments except low
259 phosphate (Figure 5). We used a *post hoc* model with growth rate as a fixed effect to
260 investigate whether the fast growing RP populations also had different chlorophyll
261 contents. Growth rate had a significant effect on chlorophyll content ($F_{1,99}$=57.86, p
262 <0.0001), with fast growing RP populations having lower chlorophyll content, and the
263 effect of growth rate was dependent on environment ($F_{4,99}$=3.85, p=0.01) and
264 resistance type ($F_{2,99}$=6.27, p =0.003). Furthermore, when growth rate was considered
265 in the model, resistance type alone had a significant effect on chlorophyll content
266 ($F_{2,6}$=5.49, p =0.04), suggesting that the growth rate of the fast growing RP
267 populations reduced chlorophyll content.
268
269 **DISCUSSION**
270
271 *Effect of environment on host resistance*
272
273 We observed no differences in susceptibility of any of the populations to OtV5 over
274 the environments tested. While the ability of the virus to lyse host cells did not depend
275 on the environment, R and RP cells had different growth responses to viral exposure.
276 There were two cases in which a resistant population repeatedly had a higher cell

277 density after exposure to OtV5 than its paired control culture that was not inoculated.
278 We speculate that this may be a response to the virus, which causes the phytoplankton
279 cells to divide more rapidly. This would be advantageous if, for example, a population
280 that was made up of mixed susceptible and resistant cells were exposed to virus – any
281 resistant cell lineages that could increase their growth rate would then take over the
282 population by overgrowing any remaining resistant cells whose growth rate was
283 unaffected by exposure to virus.
284
285 We did not detect a growth cost of resistance when R and RP populations were grown
286 in the absence or presence of OtV5 after exposure to a novel environment. We expect
287 to see a trade-off for being resistant to viral infection, because if there were no cost
288 there should be a strong selection pressure for all cells to become resistant, yet we still
289 find susceptible populations both in the laboratory and in the ocean (Thomas *et al.*,
290 2011; Clerissi *et al.*, 2012). Previous work shows that that susceptible cells can have a
291 competitive advantage against resistant cells (Lenski, 1988). Additionally, we
292 speculate that resistance to one virus strain could make these cells susceptible to other
293 OtVs. Clerissi *et al*., (2012) showed that OtVs are mainly intraspecies-specific and
294 that hosts that are the most resistant to infection can often be infected by more
295 generalist viruses. This specificity could be caused by proteins involved in adaptive
296 behavior (Clerissi *et al.*, 2012). Thus, we suggest that in addition to the abiotic
297 environment, biotic environment could play a large role in *O. tauri* resistance
298 strategy.
299
300 Since viruses are responsible for a large proportion of microbial death, there is strong
301 selection on hosts for resistance or tolerance to viral infection. There are several

suggestions to explain the paradox of how susceptible algal cells and their viruses are able to co-exist in marine environments without extinction of the host. One theory as to how viruses and their hosts are able to coexist is that there must be a cost to being resistant to infection. This is often expected to be a reduction in growth (Weinbauer, 2004), as has been observed in *Synechococcus*, in which there was a 20% reduction in fitness compared to the ancestor in resistant strains (Lennon *et al.*, 2007). Thus in the absence of viruses, resistant cells often have a lower fitness. This could lead to decreased numbers in the absence of viruses. An evolutionary "arms race" may occur when viruses and their hosts adapt reciprocally to overcome resistance and infection, respectively. We find little evidence for a cost of resistance in our study, but this may be because the laboratory environments used are missing a key aspect of the natural environment that, if present, results in a cost of resistance in *O.tauri.* Alternatively, although deviating from the standard control environment, none of the environments in this study were severely stressful, with even the low phosphate environment allowing reasonable growth. Thus, it is possible that we did not detect a growth cost because the changes to the environments used were relatively modest.

Various strategies for virus resistance have been reported in algae, including activation of programmed cell death (Bidle *et al.*, 2007), absence of metacaspase (caspase orthologues) protein expression (Bidle *et al.*, 2007), stage of the life cycle (Frada *et al.*, 2008), changes to cell surface receptor proteins (Tarutani *et al.*, 2006), colony formation (Brussaard *et al.*, 2007) and genetic mutations (Stoddard *et al.*, 2007). However, it is still unknown how *O. tauri* cells acquire their two resistance strategies. We found that short-term exposure to novel environments does not affect

326 resistance type and we did not observe any cost of resistance leading to cells losing
327 their resistance to OtV5.
328
329 *Effect of resistance type on population growth and other phenotypic traits*
330
331 We found that after one week in a novel environment, growth rate of *O. tauri*, as
332 measured by the average number of cell divisions per day over seven days, varied
333 across environments for all resistance types. RP populations had the fastest average
334 rates of cell division in most environments. All resistance types showed the same
335 environmental preferences, with average cell division rates highest in the control
336 environment. The only exception was one RP population that divided rapidly in the
337 low salt environment. The lowest growth rates were observed in the low phosphate
338 environment, which was expected since these cells were deprived of a key nutrient.
339
340 Two of the three RP populations divided more rapidly than all of the S and R
341 populations. These populations were fast growing in many environments, including
342 the control environment, suggesting that the rapid growth is a general character of
343 these two RP populations, rather than a response to stress or novelty. This faster
344 growth rate in RP populations relative to S and R populations is in contrast with
345 previous studies on *O. tauri*. Thomas *et al* (2011) detected no difference in growth
346 rate between S, R and RP cells, although competition experiments revealed a small
347 reduction of fitness in RP compared to R, and R compared to S. Our results suggest
348 that the opposite can be true. Similarly to Thomas *et al*. (2011), we did not observe a
349 fitness cost in terms of growth rate for the remaining populations since S, R and
350 normal growing RP populations had similar growth rates across environments. This

was expected, at least in the control environment, where previous studies have only been able to detect a minimal cost of resistance by using direct competitions. Surprisingly, the two fast growing RP populations could not be detected as having more rapid growth under low phosphate, however these populations responded differently in their size and chlorophyll contents.

Reduced growth rate is often observed as a cost of resistance in microbes and has been measured in several species (Lennon *et al.*, 2007; Haaber and Middelboe, 2009). Ecologically, a cost of resistance is part of "kill the winner" dynamics, where, it is hypothesized that viruses kill the faster growing (susceptible) cells, and thus provide an opportunity for slower growing (resistant) cells (Mojica and Brussaard, 2014). This role for viruses requires that there be a cost of resistance. However, here we did not detect a cost of resistance in terms of growth rate, since there was no environment in which resistant cell types grew at slower rates than S cells. In fact, we observed the opposite in two out of the three RP populations, where resistant cells grew faster than the S populations across all environments except low phosphate. In cases where resistant cells (R or RP populations) did not divide faster than susceptible ones, they divided at the same rate. Taken together, this suggests that the cost of resistance to OtVs is likely to be small or absent, and may not play into kill the winner dynamics. This opens the question of how the appearance of resistance to OtVs affects both host and viral ecology.

Environment affected cell size, whereas generally, resistance type did not. However, under low phosphate, the two fast growing RP populations were smaller than the normal growing RP populations, suggesting that under nutrient limitation these cells

were able to divide at a smaller cell size. Smaller phytoplankton cell size is often selected for in nutrient limited environments since smaller cells have a larger surface area to volume ratio and a thinner diffusion boundary layer, thus facilitating nutrient uptake (Finkel *et al.*, 2010; Peter and Sommer, 2015). Although fast growing RP populations in this selection environment were smaller than the normal growing RP population, their cell size was not different from the fast growing RP populations in the other environments. The control was the only environment in which fast growing RP populations were larger than the normal growing populations, indicating that there may be a (direct or indirect) fitness benefit associated with the increased size of the RP type under control conditions.

In contrast to previous studies, all populations in the low phosphate environment, except fast growing RP, increased in cell size. Cell division of larger phytoplankton cells requires greater nutrient concentrations, which can decrease the division rate. Since cells in the low phosphate environment had a reduced growth rate in terms of cell divisions, this could have resulted in cells that reached a larger volume even though the environment was phosphate-poor. It has previously been suggested that increasing algal cell size, and thus the volume to surface area ratio, can facilitate reduced phosphorus uptake under phosphate-limited conditions, and that this adaptation response may be more favourable than decreasing cell size (Šupraha *et al.*, 2015). A common response of coccolithophores to phosphate limitation is reduced growth rate and increased cell size (Šupraha *et al.*, 2015).

Smaller phytoplankton cells have often been observed growing at higher temperatures in natural environments, which is thought to arise from the temperature-size rule (e.g.

401 Atkinson *et al.*, 2003; Morán *et al.*, 2010). These studies used large temperature
402 ranges, but there was no effect of the modest increase in temperature on cell size in
403 this study. Smaller cells have also been reported to cope better with both light
404 limitation and light saturation compared to larger cells due to a reduction in internal
405 shading (Geider *et al.*, 1986; Raven, 1998; Finkel *et al.*, 2010). We found no
406 significant difference in cell size under low light, although there was a non-significant
407 trend for cells to be slightly larger in low than under control light.
408
409 Environment was found to have a significant effect on chlorophyll content per cell
410 volume, whereas resistance type alone had no effect. We observed lower chlorophyll
411 per cell volume in all environments compared to the control except high temperature.
412 Although resistance type alone did not have an effect, growth rate had a significant
413 effect on chlorophyll content per cell volume when included in the model and normal
414 growing S, R and RP cells in the control and high temperature environments had the
415 highest chlorophyll levels across all environments. In contrast, fast growing RP
416 populations showed no significant difference in chlorophyll content per cell volume
417 across all five environments. All populations had their lowest growth rates in the low
418 phosphate environment and cells in this environment had the lowest chlorophyll
419 content, except for fast growing RP populations. Fast growing RP populations had
420 lower chlorophyll content than the normal growing RP population in all environments
421 except for low phosphate.
422
423 One experiment using cultures of different phytoplankton groups found that
424 chlorophyll content was lower during both nitrogen and phosphorus depletion
425 (Riemann *et al.*, 1989). Additionally, phytoplankton cells grown under low nutrients

have been observed to decrease their photosynthesis rates (Litchman *et al.*, 2003; Spilling *et al.*, 2015). This may be due to the cells allocating resources to synthesizing chloroplasts under nutrient limitation. In our study the control environment was the preferred one, and it is possible that cells were unable to synthesise large quantities of chlorophyll in the other (less permissive) environments since their energy was allocated to growth. It is possible that under elevated temperature, the metabolism of *O. tauri* was increased, leading the cells to synthesise more chlorophyll. Temperature did not affect chlorophyll a content in diatoms (Sigaud and Aidar, 1993). Salinity appears to affect different phytoplankton species differently, with some species showing no change in chlorophyll content across a range of salinities, and others having higher chlorophyll contents at the optimum salinity for growth (McLachlan, 1961; Sigaud and Aidar, 1993).

Concluding remarks

Resistance of microbes to virus infection often comes at a cost, with one common observation being a reduction in growth compared to susceptible cells in the population. In this study, our aim was to measure resistance to viruses in *O. tauri* across different environments and to determine whether the magnitude of a cost of resistance depends on environmental context. We did not observe a cost of resistance as measured by cell divisions, cell size or chlorophyll content in the present study. Growth rates of *O. tauri* were reduced when grown in low phosphate, however this did not affect the ability of OtV5 to lyse susceptible cells in this environment. Additionally, although growth rates were lower than the controls in high temperature, low light and low salinity, OtV5 still caused cell lysis of susceptible cells. Indeed,

some populations that were tolerant to infection (RP populations) had evolved high growth rates, and some RP populations also increased their growth rates after exposure to viruses. Both observations suggest that resistance strategy could have interesting ecological consequences by changing the relative fitness of different populations.

EXPERIMENTAL PROCEDURES

Susceptible and resistant populations used in this experiment

O. tauri populations were obtained from N. Grimsley, Observatoire Océanologique, Banyuls-sur-Mer. Three susceptible populations (NG'2, NG'3 and NG'4), three resistant producer populations (NG'10, NG'16 and NG27) and three resistant populations (NG5, NG'13 and NG26) were used in this study. We used three biological replicates for each population in each environment. All populations were derived from a single clone of *O. tauri* (RCC 4221) and therefore had the same starting genotype (see Thomas *et al*., 2011). All populations have since been maintained separately. All RP populations were tested for viral production prior to the start of the experiment (See Supporting Information Figure S1).

Culturing conditions

Populations were grown in batch culture. Culture medium was prepared using 0.22 μm filtered Instant Ocean artificial seawater (salinity 30 ppt) aerated with 400 ppm CO_2 and supplemented with Keller and f/2 vitamins. Control cultures were maintained in a 14:10 light:dark cycle at 85 μmol photon m^{-2} s^{-1} and at a constant temperature of 18°C (Table 1).

For the selection experiments, *O. tauri* populations were grown without exposure to viruses in the control environment and four selection environments. The selection regimes used were high temperature, low light, low phosphate and low salinity (Table

1). Cultures were acclimated in each selection environment for one week, followed by one week of growth in each environment.

Environment	Control	Treatment
Phosphate (μM)	10	5
Salinity (ppt)	30	25
Light (μmol m^{-2} s^{-1})	85	60
Temperature (°C)	18	20

For the low phosphate environment, phosphate was reduced by preparing Keller media with only half the amount of β-glycerophosphate that would normally be used. Although the phosphate concentration in the low phosphate environment is not low compared to natural seawater (0.01-2.99 μmol l^{-1} in the Leucate lagoon where *O. tauri* and OtV5 inhabit (Clerissi *et al.*, 2014)), it is low compared to the control media in which the populations had been maintained prior to the experiment. For culture medium with a lower salinity than the control, Instant Ocean was added to reach a salinity of 25 ppt. For the low light condition, culture flasks were wrapped in 0.15 neutral density foil to give a light intensity of 1000 lux. Cultures in the high temperature condition were maintained on a heat mat (Exo Terra Heat Wave substrate heat mat) set at 20°C.

The effect of viral exposure on cell division rates

Following one week of growth in the selection environment, each sample was inoculated with a fresh suspension of OtV5 particles to test whether it was susceptible

501 or resistant to the virus. Samples were tested by inoculating 1 ml cell culture at a
502 density of 10^5 with 10 µl OtV5 in 48-well plates with three replicates for each sample.
503 Controls that were not inoculated with viruses were used as a control for cell growth.
504 Cell density was measured using a FACSCanto flow cytometer 3 days after
505 inoculation.
506
507 *Population growth of susceptible and resistant populations across different*
508 *environments*
509
510 Following the acclimation period, average cell densities per day of all cultures were
511 measured over one week of growth in each environment. Cells were counted using a
512 BD FACSCanto II (BD Biosciences) flow cytometer before the first transfer and after
513 seven days of growth. Each population was counted in triplicate. The cell counts were
514 converted to cells per millilitre and the number of divisions per day was calculated
515 using equation (1).
516
517 (1) $$\mu\,(d^{-1}) = \frac{\log_2\left(\frac{N_t}{N_0}\right)}{t - t_0}$$
518
519 where N_t and N_0 are the cell densities (cells ml^{-1}) at times t_1 and t_0 (days). This
520 measures the average number of cell divisions per ancestor over a single growth cycle
521 and allows a comparison of offspring production between environments (Brennan and
522 Collins, 2015). This is useful if different environments produce different growth
523 curves since populations with different growth strategies can be compared. This
524 calculation is also not sensitive to small differences in N_0, which is important if the

525 population size reached during the acclimation period differs between environments
526 or resistance types.
527
528 *Cell size and chlorophyll content of populations with different resistance types across*
529 *environments*
530
531 Cell size and relative chlorophyll content per cell volume were determined using a
532 FACSCanto flow cytometer. Cell size was inferred from FSC (forward scatter), which
533 was calibrated using beads of known sizes (1μm, 3μm and 6.6μm). Chlorophyll
534 fluorescence was inferred by measuring PerCP-Cy5.5 emission with excitation at
535 488nm. Relative chlorophyll was analysed by taking the average chlorophyll
536 fluorescence for all susceptible populations in the control environment and setting this
537 to a value of 1, with chlorophyll measurements of all populations relative to this
538 value.
539
540 *Statistical analysis*
541
542 Data were analysed with linear mixed effects models using the statistical package
543 nlme in R (version 3.2.0) to identify differences in growth rates between the different
544 environments after one week of growth and after virus inoculation. Environment and
545 resistance type were fixed effects when analyzing growth under different
546 environments, and environment, resistance type and treatment were fixed effects
547 when analyzing virus inoculation under different environments. Population was a
548 random effect in both models.
549

Post hoc mixed effects models were used to examine whether growth rate had an effect on cell size and chlorophyll content in cells. Environment, resistance type and growth rate (cells divisions per day) were set as fixed effects with populations as the only random effect.

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REFERENCES

Atkinson, D., Ciotti, B.J., and Montagnes, D.J.S. (2003) Protists decrease in size linearly with temperature: ca. 2.5% degrees C(-1). *Proc. Biol. Sci.* **270**: 2605–11.

Avrani, S. and Lindell, D. (2015) Convergent evolution toward an improved growth rate and a reduced resistance range in Prochlorococcus strains resistant to phage. *Proc. Natl. Acad. Sci. U. S. A.* **112**: E2191–200.

Bellec, L., Grimsley, N., Derelle, E., Moreau, H., and Desdevises, Y. (2010) Abundance, spatial distribution and genetic diversity of Ostreococcus tauri viruses in two different environments. *Environ. Microbiol. Rep.* **2**: 313–21.

Bidle, K.D., Haramaty, L., Barcelos E Ramos, J., and Falkowski, P. (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophore, Emiliania huxleyi. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 6049–54.

Blanc-Mathieu, R., Verhelst, B., Derelle, E., Rombauts, S., Bouget, F.-Y., Carré, I., et

575 al. (2014) An improved genome of the model marine alga Ostreococcus tauri
576 unfolds by assessing Illumina de novo assemblies. *BMC Genomics* **15**: 1103.
577 Bohannan, B.J.M., Kerr, B., Jessup, C.M., Hughes, J.B., and Sandvik, G. (2002)
578 Trade-offs and coexistence in microbial microcosms. *Antonie van Leeuwenhoek,*
579 *Int. J. Gen. Mol. Microbiol.* **81**: 107–115.
580 Børsheim, K.Y. (1993) Native marine bacteriophages. *FEMS Microbiol. Lett.* **102**:
581 141–159.
582 Bratbak, G., Egge, J.K., and Heldal, M. (1993) Viral mortality of the marine alga
583 Emiliania huxleyi (Haptophyceae) and termination of algal blooms. *Mar. Ecol.*
584 *Prog. Ser.* **93**: 39–48.
585 Bratbak, G., Jacobsen, A., Heldal, M., Nagasaki, K., and Thingstad, F. (1998) Virus
586 production in Phaeocystis pouchetii and its relation to host cell growth and
587 nutrition. *Aquat. Microb. Ecol.* **16**: 1–9.
588 Brennan, G. and Collins, S. (2015) Growth responses of a green alga to multiple
589 environmental drivers. *Nat. Clim. Chang.* **5**: 892–897.
590 Brussaard, C.P.D., Bratbak, G., Baudoux, A.C., and Ruardij, P. (2007) Phaeocystis
591 and its interaction with viruses. *Biogeochemistry* **83**: 201–215.
592 Bruussard, C.P. (2004) Viral control of phytoplankton populations - a review. *J.*
593 *Eukaryot. Microbiol.* **51**: 125–138.
594 Chen, S., Gao, K., and Beardall, J. (2014) Viral attack exacerbates the susceptibility
595 of a bloom-forming alga to ocean acidification. *Glob. Chang. Biol.* 629–636.
596 Clerissi, C., Desdevises, Y., and Grimsley, N. (2012) Prasinoviruses of the marine
597 green alga Ostreococcus tauri are mainly species specific. *J. Virol.* **86**: 4611–9.
598 Clerissi, C., Grimsley, N., Subirana, L., Maria, E., Oriol, L., Ogata, H., et al. (2014)
599 Prasinovirus distribution in the Northwest Mediterranean Sea is affected by the

environment and particularly by phosphate availability. *Virology* **466-467**: 146–157.

Danovaro, R., Corinaldesi, C., Dell'Anno, A., Fuhrman, J. a., Middelburg, J.J., Noble, R.T., and Suttle, C. a. (2011) Marine viruses and global climate change. *FEMS Microbiol. Rev.* **35**: 993–1034.

Derelle, E., Ferraz, C., Escande, M.-L., Eychenié, S., Cooke, R., Piganeau, G., et al. (2008) Life-cycle and genome of OtV5, a large DNA virus of the pelagic marine unicellular green alga Ostreococcus tauri. *PLoS One* **3**: e2250.

Finkel, Z. V., Beardall, J., Flynn, K.J., Quigg, A., Rees, T.A. V, and Raven, J.A. (2010) Phytoplankton in a changing world: Cell size and elemental stoichiometry. *J. Plankton Res.* **32**: 119–137.

Frada, M., Probert, I., Allen, M.J., Wilson, W.H., and de Vargas, C. (2008) The "Cheshire Cat" escape strategy of the coccolithophore Emiliania huxleyi in response to viral infection. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 15944–9.

Frickel, J., Sieber, M., and Becks, L. (2016) Eco-evolutionary dynamics in a coevolving host-virus system. *Ecol. Lett.* **19**: 450–459.

Geider, R., Platt, T., and Raven, J. (1986) Size dependence of growth and photosynthesis in diatoms: a synthesis. *Mar. Ecol. Ser.* **30**: 93–104.

Haaber, J. and Middelboe, M. (2009) Viral lysis of Phaeocystis pouchetii: implications for algal population dynamics and heterotrophic C, N and P cycling. *Isme J* **3**: 430–441.

Jacquet, S. and Bratbak, G. (2003) Effects of ultraviolet radiation on marine virus-phytoplankton interactions. *FEMS Microbiol. Ecol.* **44**: 279–289.

Larsen, J.B., Larsen, a., Thyrhaug, R., Bratbak, G., and Sandaa, R. -a. (2007) Marine viral populations detected during a nutrient induced phytoplankton bloom at

625 elevated pCO_2 levels. *Biogeosciences Discuss.* **4**: 3961–3985.
626 Lennon, J.T., Khatana, S.A.M., Marston, M.F., and Martiny, J.B.H. (2007) Is there a
627 cost of virus resistance in marine cyanobacteria? *ISME J.* **1**: 300–312.
628 Lenski, R.E. (1988) Experimental studies of pleiotropy and epistasis in Escherichia
629 coli. I. variation in competitive fitness among mutants resistant to virus T4.
630 *Evolution (N. Y).* **42**: 425–432.
631 León, M. and Bastías, R. (2015) Virulence reduction in bacteriophage resistant
632 bacteria. *Front. Microbiol.* **6**: 1–7.
633 Litchman, E., Steiner, D., and Bossard, P. (2003) Photosynthetic and growth
634 responses of three freshwater algae to phosphorus limitation and daylength.
635 *Freshw. Biol.* **48**: 2141–2148.
636 Maat, D., Crawfurd, K., Timmermans, K., and Brussard, C. (2014) Elevated CO2 and
637 phosphate limitation favor Micromonas pusilla through stimulated growth and
638 reduced viral impact. *Appl. Environ. Microbiol.*
639 Marston, M.F., Pierciey, F.J., Shepard, A., Gearin, G., Qi, J., Yandava, C., et al.
640 (2012) Rapid diversification of coevolving marine Synechococcus and a virus.
641 *Pnas* **109**: 4544–4549.
642 McLachlan, J. (1961) The effect of salinity on growth and chlorophyll content in
643 representative classes of unicellular marine algae. *Can. J. Microbiol.* **7**: 399–406.
644 Mojica, K.D.A. and Brussaard, C.P.D. (2014) Factors affecting virus dynamics and
645 microbial host-virus interactions in marine environments. *FEMS Microbiol.*
646 *Ecol.* **89**: 495–515.
647 Morán, X.A.G., López-Urrutia, Á., Calvo-Díaz, A., and Li, W.K.W. (2010)
648 Increasing importance of small phytoplankton in a warmer ocean. *Glob. Chang.*
649 *Biol.* **16**: 1137–1144.

650 Nagasaki, K. and Yamaguchi, M. (1998) Effect of temperature on the algicidal
651 activity and the stability of HaV (Heterosigma akashiwo virus). *Aquat. Microb.*
652 *Ecol.* **15**: 211–216.
653 Peter, K.H. and Sommer, U. (2015) Interactive effect of warming, nitrogen and
654 phosphorus limitation on phytoplankton cell size. *Ecol. Evol.* **5**: 1011–1024.
655 Raven, J.A. (1998) The twelfth Tansley Lecture. Small is beautiful: The
656 picophytoplankton. *Funct. Ecol.* **12**: 503–513.
657 Riemann, B., Simonsen, P., and Stensgaard, L. (1989) The carbon and chlorophyll
658 content of phytoplankton from various nutrient regimes. *J. Plankton Res.* **11**:
659 1037–1045.
660 Seed, K.D., Faruque, S.M., Mekalanos, J.J., Calderwood, S.B., Qadri, F., and Camilli,
661 A. (2012) Phase Variable O Antigen Biosynthetic Genes Control Expression of
662 the Major Protective Antigen and Bacteriophage Receptor in Vibrio cholerae O1.
663 *PLoS Pathog.* **8.**:
664 Sigaud, T.C.S. and Aidar, E. (1993) Salinity and temperature effects on the growth
665 and chlorophyll-a content of some planktonic aigae. *Bol. do Inst. Ocean.* **41**: 95–
666 103.
667 Spilling, K., Ylöstalo, P., Simis, S., and Seppälä, J. (2015) Interaction effects of light,
668 temperature and nutrient limitations (N, P and Si) on growth, stoichiometry and
669 photosynthetic parameters of the cold-water diatom Chaetoceros wighamii. *PLoS*
670 *One* **10**: 1–18.
671 Stoddard, L.I., Martiny, J.B.H., and Marston, M.F. (2007) Selection and
672 characterization of cyanophage resistance in marine Synechococcus strains.
673 *Appl. Environ. Microbiol.* **73**: 5516–22.
674 Šupraha, L., Gerecht, A.C., Probert, I., and Henderiks, J. (2015) Eco-physiological

675 adaptation shapes the response of calcifying algae to nutrient limitation. *Sci. Rep.*
676 **5**: 16499.
677 Tarutani, K., Nagasaki, K., and Yamaguchi, M. (2006) Virus adsorption process
678 determines virus susceptibility in Heterosigma akashiwo (Raphidophyceae).
679 *Aquat. Microb. Ecol.* **42**: 209–213.
680 Thomas, R., Grimsley, N., Escande, M.-L., Subirana, L., Derelle, E., and Moreau, H.
681 (2011) Acquisition and maintenance of resistance to viruses in eukaryotic
682 phytoplankton populations. *Environ. Microbiol.* **13**: 1412–20.
683 Weinbauer, M.G. (2004) Ecology of prokaryotic viruses. **28**: 127–181.
684 Wells, L.E. and Deming, J.W. (2006) Effects of temperature, salinity and clay
685 particles on inactivation and decay of cold-active marine Bacteriophage 9A.
686 *Aquat. Microb. Ecol.* **45**: 31–39.
687 Weynberg, K.D., Allen, M.J., Ashelford, K., Scanlan, D.J., and Wilson, W.H. (2009)
688 From small hosts come big viruses: the complete genome of a second
689 Ostreococcus tauri virus, OtV-1. *Environ. Microbiol.* **11**: 2821–39.
690 Weynberg, K.D., Allen, M.J., Gilg, I.C., Scanlan, D.J., and Wilson, W.H. (2011)
691 Genome sequence of Ostreococcus tauri virus OtV-2 throws light on the role of
692 picoeukaryote niche separation in the ocean. *J. Virol.* **85**: 4520–9.
693 Wilhelm, S.W. and Suttle, C.A. (1999) Viruses and nutrient cycles in the sea.
694 *Bioscience* **49**: 781–788.
695
696
697 **TABLE AND FIGURE LEGENDS**
698

699 Table 1. A comparison of the control environment and the environment treatments
700 that were used for each environmental condition in this study.
701
702 Figure 1. Mean cell densities ml^{-1} of resistant (R), resistant producer (RP) and
703 susceptible (S) *O. tauri* cells. Inoculated = cells inoculated with OtV5, Not inoculated
704 = control cultures that were grown for the same amount of time, but not inoculated
705 with OtV5. The dashed line represents the starting densities of the cultures at 10^5 cells
706 ml^{-1}. There were three biological replicates for each populations. Boxes represent the
707 interquartile range with the median indicated as the thick black line inside the box,
708 and whiskers extend to the highest and lowest values within 1.5 × the inter-quartile
709 range from the edge of the box. Outlier data beyond the end of the whiskers are
710 plotted as points.
711
712 Figure 2. Mean growth rates, measured as average number of cell divisions per day
713 over 7 days, of susceptible (S), resistant (R) and resistant producer (RP) *O. tauri* cells
714 grown in five environments in the absence of OtV5. There were three populations for
715 each resistance type, with three biological replicates for each population. Boxes
716 represent the interquartile range with the median indicated as the thick black line
717 inside the box, and whiskers extend to the highest and lowest values within 1.5 × the
718 inter-quartile range from the edge of the box. Outlier data beyond the end of the
719 whiskers are plotted as points.
720
721 Figure 3. Ranked environment by average cell divisions per day over 7 days (±
722 SEM) for susceptible (S), resistant (R) and resistant producer (RP and RPfast) cells.
723 Environments were ranked in order from best to worst for each resistance type based

724 on growth rate in the absence of OtV5, where 1 is the environment with the highest
725 growth rate. Fast and normal growing resistant producers have been plotted separately
726 for visual purposes.
727
728 Figure 4. Mean cell size for susceptible (S), resistant (R) and resistant producer (RP)
729 cells after seven days of growth in the absence of viruses in five environments. There
730 were three populations for each resistance type, with three biological replicates for
731 each populations. Boxes represent the interquartile range with the median indicated as
732 the thick black line inside the box, and whiskers extend to the highest and lowest
733 values within 1.5 × the inter-quartile range from the edge of the box. Outlier data
734 beyond the end of the whiskers are plotted as points.
735
736 Figure 5. Mean relative chlorophyll to cell size for susceptible (S), resistant (R) and
737 resistant producer (RP) cells after seven days of growth in the absence of viruses in
738 five environments. There were three populations for each resistance type, with three
739 biological replicates for each population. Boxes represent the interquartile range with
740 the median indicated as the thick black line inside the box, and whiskers extend to the
741 highest and lowest values within 1.5 × the inter-quartile range from the edge of the
742 box. Outlier data beyond the end of the whiskers are plotted as points.
743
744 Table S1. ANOVA results of a linear mixed effects model to analyse interaction
745 effects of environment, resistance type and treatment (with or without OtV5
746 inoculation) on *O. tauri* cell density. Population was a random effect.
747

748 Table S2. ANOVA results of a linear mixed effects model to analyse interaction
749 effects of environment, resistance type, treatment (with or without OtV5 inoculation)
750 and growth rate (fast RP or normal) on *O. tauri* growth rate, as measured by cell
751 divisions per day. Population was set as a random effect in all models.
752
753 Table S3. Ranked environments by fitness as measured by cell divisions per day for
754 each resistance type. Environments were ranked in order from best to worst, where 1
755 is the environment with the highest growth rate. Fast and normal growing resistant
756 producers were ranked separately to compare slopes.
757
758 Figure S1. Mean cell densities ml^{-1} (±SEM) of *O. tauri* strain RCC4221 three days
759 after inoculation with supernatant from Resistant Producing populations (NG'10,
760 NG'16 and NG27). To ensure that the RP populations being used in this experiment
761 were producing infectious viruses and releasing them to their external surroundings,
762 we used the supernatant of these strains to infect susceptible *O. tauri* cells.
763 Populations NG'10, NG'16 and NG'27 were aliquoted into 2ml Eppendorf tubes and
764 centrifuged at 8000 × g for 15 min. Next, 400 µl of supernatant was carefully
765 removed without drawing up any cells from the pellet at the bottom of the tube, and
766 used to inoculate 1 ml of susceptible *O. tauri* strain RCC4221. Eight replicates were
767 performed. A positive control was performed using known OtV5, and a negative
768 control was performed by adding Keller media. Controls were performed in
769 quadruplicate. Cells were left to grow for 3 days after which their densities were
770 measured using a FACSCanto flow cytometer. We observed cell lysis resulting from
771 inoculation with supernatant from all three RP populations, showing that there was
772 active virus in the media taken from these cultures.